PHOSPHOLIPASE A_2 (PLA_2) REGULATES NEUROEXOCYTOSIS TO COUNTERACT BOTULINUM TOXIN A (BONT/A) POISONING

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ABSTRACT

In this study, BoNT/A is one of the most serious biological threats faced by the U. S. military and allied forces. Previously we reported that in nerve growth factor (NGF)-differentiated PC12 cells, arachidonic acid (AA) release is associated with acetylcholine (ACh) release and BoNT/A inhibits both. We report the effect of PLA_2 over expression on inhibition of ACh exocytosis due to BoNT/A light chain (LC) in PC12 cells. Over expression of PLA_2 alone augmented the stimulated release of ACh and AA. PLA_2 over expression also effectively prevented the inhibition of stimulated ACh and AA release due to BoNT/A LC.

INTRODUCTION

Botulinum toxin type A (BoNT/A) acts by blocking Ca^{2+}-dependent ACh release (neuroexocytosis) at peripheral neuromuscular junctions, sometimes causing fatal neuromuscular paralysis. The clonal pheochromocytoma PC12 cell line was used to study the biochemical mechanisms of action of BoNT/A. To explain the mechanism of action of BoNT/A, we previously reported that when NGF differentiated PC12 cells are stimulated with high K^+ (80 mM), AA and ACh are co-released and BoNT/A inhibits both release processes. (Ray, P. *et al*., 1993, J. Biol. Chem., 268, 11057-11064). Stimulus-induced ACh release and AA release are inhibited in the presence of Ca^+-dependent PLA_2 inhibitors (AACOCF3, DEDA). On the contrary, PLA_2 activators such as mastoparan can cause ACh release in cells (Ray, P. *et al*., 1997, NeuroReport, 8, 2271-2274).

PLA2 acts on membrane phospholipids to generate AA and lysophosphatidic acid (LPA). AA acts as a fusogen to induce exocytosis. LPA activates Rho GTPases, which have been implicated in mechanisms correlating actin cytoskeletal organization and exocytosis. In this study, we show that PLA2 over expression prevented the inhibition of stimulated ACh and AA release due to BoNT/A LC.

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METHODS

CELL CULTURE

PC12 cells (CLONTECH) were grown in 75-cm^2 tissue culture flasks in DMEM (GIBCO) containing 5 % (v/v) FBS 10 % (v/v) donor horse serum (CLONTECH), streptomycin 100 µg/ml and 100 units/ml penicillin G sodium (Sigma). The cells (1.5 X 10^6) were incubated at 37ºC in a humidified atmosphere of 90% air 10% CO_2. After 3 days the cells were differentiated with nerve growth factor (NGF, 50 ng/ml growth medium) for 4 days.

$SPLA_2$ OVER EXPRESSION IN PC12 CELLS

The levels of $sPLA_2$ in differentiated PC12 cells after transfection with pTracer-PLA_2 was analysed by immunoprecipitation and Western blotting analysis using rabbit antiserum against $sPLA_2$ (Cayman Chemical).

BONT/A LC OVER EXPRESSION IN PC12 CELLS

Expression of BoNT/A LC after transfection was analysed by Western blotting with use of GFP monoclonal antibody (CLONTECH).

SNAP-25 DETECTION

SNAP-25 was visualized by SNAP-25 antibody and TRITC conjugated IgG.

ARACHIDONIC ACID (AA) RELEASE ASSAY

Arachidonic acid release assay was performed by modification of the method of Ray.P et al. as described (J. Biol. Chem., 268, 11057-11064).

ACETYLCHOLINE (ACH) RELEASE ASSAY

Acetylcholine release assay was performed by modification of method of Ray.P et al. as described (J. Biol. Chem., 268, 11057-11064).

RESULTS

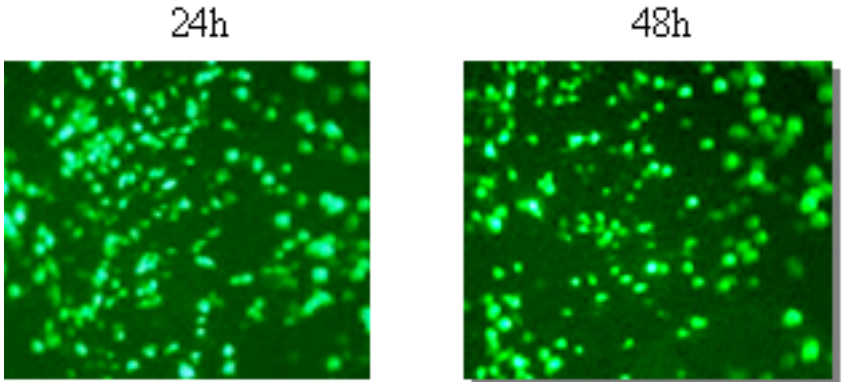

Figure 1. Transfection efficiency of PC 12 cells by pEGFP-BoNT/A LC

The transfection efficiency (>70%) was calculated twenty-four hours and forty-eight hours after transfection with pEGFP-BoNT/A LC by fluorescence microscope. Images were taken twenty-four hours and forty-eight hours after transfection

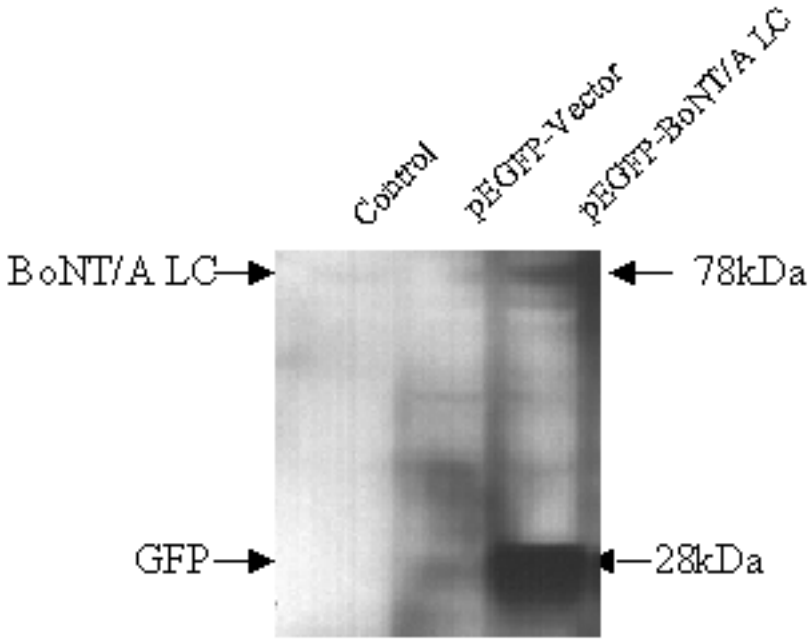

Figure 2. BoNT/A LC over expression in PC12 cells

Twenty-four hours after transfection of differentiated PC 12 cells with pEGFP-BoNT/A LC, the expression level of LC was confirmed by Western blotting using of GFP antibody. Control: No GFP was expressed; Transfection with pEGFP vector alone: GFP (28 kDa) was expressed; Transfection with pEGFP-BoNT/A LC: GFP-fused LC (78 kDa) was expressed.

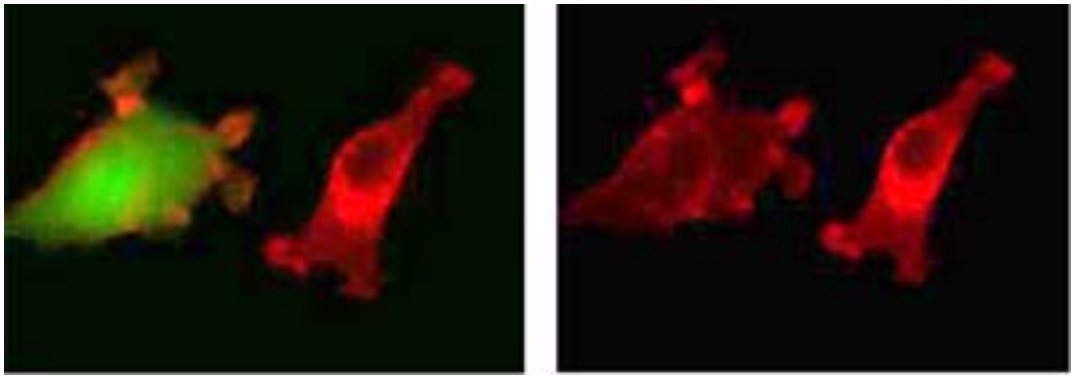

Figure 3. Effect of transfection of BoNT/A LC on SNAP-25 degradation

Twenty-four hours after transfection of differentiated PC12 cells with pEGFP-BoNT/A LC, SNAP-25 was visualized by SNAP-25 antibody and TRITC conjugated IgG. There was a marked degradation of SNAP-25 (red) in LC expressing cells (green), but no degradation was seen in untransfected cells.

Figure 4. $sPLA_2$ over expression

Immunoprecipitation followed by Western blotting analysis with $sPLA_2$ antibody, showed that transfection with $sPLA_2$ over expressed $sPLA_2$ (14 kDa). However, control cell (no transfection) or vector alone transfection did not express detectable amount of the protein.

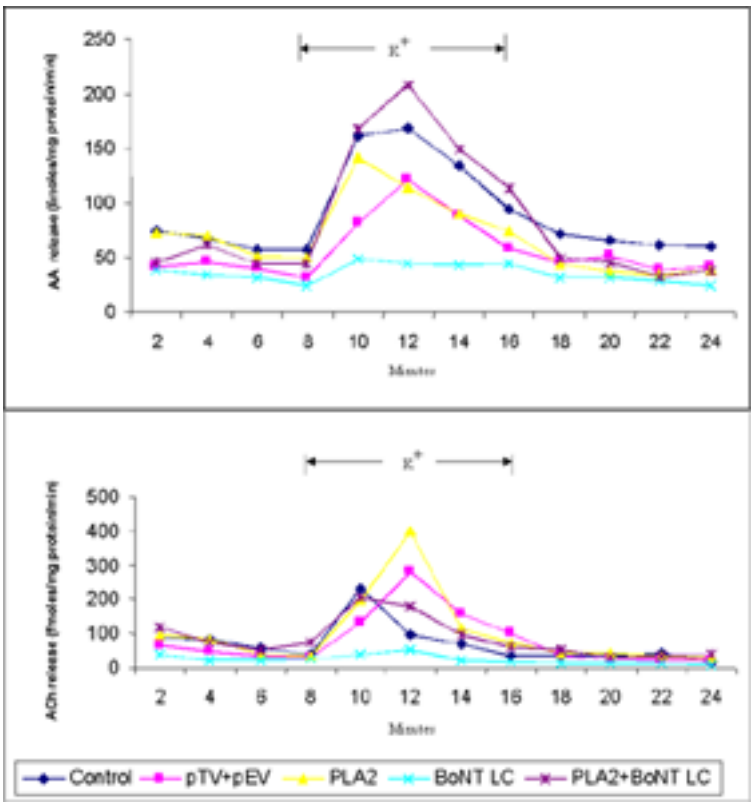

Figure 5. Effect of cotransfection of BoNT/A LC and PLA_2 on AA and ACh release

NGF-differentiated PC12 cells were transiently transfected with either pEGFP (pEV)-BoNT/A LC or cotransfected with pTracer (pTV)-sPLA_2. Over expression of PLA_2 in these BoNT/A LC transfected cells effectively prevented the inhibition of stimulated (by high K+) ACh and AA release due to BoNT/A LC.

CONCLUSION

- PLA_2 mediated mechanisms may regulate presynaptic ACh exocytosis.
- BoNT/A inhibits neuroexocytosis by targeting these mechanisms.
- Manipulating PLA_2 mechanisms in neuroexocytosis may serve as prospective strategies to counteract BoNT/A poisoning.

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